Methylmercury uptake and degradation by methanotrophs

Xia Lu, Wenyu Gu, Linduo Zhao, Muhammad Farhan Ul Haque, Alan A. DiSpirito, Jeremy D. Semrau, Baohua Gu

Methylmercury (CH$_3$Hg$^+$) is a potent neurotoxin produced by certain anaerobic microorganisms in natural environments. Although numerous studies have characterized the basis of mercury (Hg) methylation, no studies have examined CH$_3$Hg$^+$ degradation by methanotrophs, despite their ubiquitous presence in the environment. We report that some methanotrophs, such as Methylosinus trichosporium OB3b, can take up and degrade CH$_3$Hg$^+$ rapidly, whereas others, such as Methyllococcus capsulatus Bath, can take up but not degrade CH$_3$Hg$^+$. Demethylation by M. trichosporium OB3b increases with increasing CH$_3$Hg$^+$ concentrations but was abolished in mutants deficient in the synthesis of methanobactin, a metal-binding compound used by some methanotrophs, such as M. trichosporium OB3b. Furthermore, addition of methanol (>5 mM) as a competing one-carbon (C1) substrate inhibits demethylation, suggesting that CH$_3$Hg$^+$ degradation by methanotrophs may involve an initial bonding of CH$_3$Hg$^+$ by methanobactin followed by cleavage of the C–Hg bond in CH$_3$Hg$^+$ by the methanol dehydrogenase. This new demethylation pathway by methanotrophs indicates possible broader involvement of C1-metabolizing aerobes in the degradation and cycling of toxic CH$_3$Hg$^+$ in the environment.

INTRODUCTION

Methylmercury (CH$_3$Hg$^+$) toxin is predominantly produced by certain anaerobic microorganisms (for example, Desulfovibrio desulfuricans ND132 and Geobacter sulfurreducens PCA) having two key genes (hgcA and hgcB) necessary for converting inorganic mercury (Hg) to CH$_3$Hg$^+$ (1–3). It can accumulate and biomagnify at high levels in fish as well as in rice grains, and human consumption can cause neurological damage (4–7). Our understanding of the mechanisms responsible for Hg methylation has greatly improved recently through the identification of the genetic basis (1–3) and factors affecting Hg methylation (8–12). However, net CH$_3$Hg$^+$ levels in the environment depend on two competing biological processes—CH$_3$Hg$^+$ production and demethylation (13–17), although demethylation can also take place photochemically in surface waters (18).

To date, much attention has focused on Hg methylation, but fewer studies have examined microbial demethylation, except the process mediated by the mer operon (17, 19), in which demethylation is carried out by an organomercurial lyase (MerB). MerB degrades the methyl group to form methane (CH$_4$) and Hg(II), whereas a mercuric reductase (MerA) reduces the released Hg(II) to volatile elemental Hg(0) (7, 13, 20). However, only certain aerobic prokaryotes have this CH$_3$Hg$^+$ degradation pathway. mer-mediated pathway is operative only at extremely high Hg concentrations (that is, micromolar) (7, 17, 21), conditions that are largely irrelevant to most natural waters and sediments, where Hg or CH$_3$Hg$^+$ concentrations are usually at picomolar to low nanomolar ranges (7, 17). In addition, in vitro experiments with the isolated MerB enzyme showed that demethylation by MerB occurs generally at above neutral pH conditions, with an optimal pH of ~10 (21).

However, degradation of CH$_3$Hg$^+$ has been observed in anoxic sediments and in a limited number of pure cultures at relatively low Hg concentrations (for example, nanomolar) (14–17, 22). Anaerobic sulfate reducers and methanogens are thought to be primarily responsible for this oxidative demethylation because Hg(II), CH$_4$, and carbon dioxide (CO$_2$) have been identified as major products (14–16). Despite the finding of CH$_4$ and CO$_2$ evolution, methanotrophs are not considered important players in the oxidative demethylation of CH$_3$Hg$^+$. The possible involvement of methanotrophs has never been directly tested, and the bacteria involved and pathways leading to oxidative demethylation remain unexplored.

Methanotrophs can thrive under a wide range of redox conditions, particularly at the oxic-anoxic interface where CH$_4$ and CH$_3$Hg$^+$ are commonly observed (23–25). They are widespread and found in diverse locations, such as freshwater and marine sediments, bogs, forest and agricultural soils, and volcanic soils (26, 27). Many methanotrophs also produce an extracellular metal-binding peptide called methanobactin that has been shown to bind CH$_3$Hg$^+$ (28, 29). In addition, most methanotrophs can use methanol as a one-carbon (C1) growth substrate, and some can also grow on methylamine (24, 30), and we therefore hypothesized that at least some methanotrophs can take up and possibly degrade CH$_3$Hg$^+$.

RESULTS AND DISCUSSION

CH$_3$Hg$^+$ uptake and degradation were first examined in representative strains of α-(Methylosinus trichosporium OB3b) and γ-proteobacterial (Methylococcus capsulatus Bath) methanotrophs. Both methanotrophs were found to sorb substantial amounts of CH$_3$Hg$^+$, with M. trichosporium OB3b showing slightly higher sorption affinity and kinetics than M. capsulatus Bath (Fig. 1A). Within 1 hour, ~95% of the CH$_3$Hg$^+$ was sorbed or associated with M. trichosporium OB3b, whereas only ~65% was associated with M. capsulatus Bath cells, although the sorption increased to ~85% on M. capsulatus Bath cells in 4 hours. Analyses of Hg species distributions indicated that a large percentage of the CH$_3$Hg$^+$ was internalized or taken up by both M. trichosporium OB3b and M. capsulatus Bath cells in 4 hours, leaving only a small percentage of the CH$_3$Hg$^+$ in solution (Fig. 1B). These results are in contrast to the rapid export and little sorption of CH$_3$Hg$^+$ observed with known mercury methylators, such as D. desulfuricans ND132 (10, 31, 32), suggesting that both M. trichosporium OB3b and M. capsulatus Bath have a high affinity to sorb or take up CH$_3$Hg$^+$.
We found that, with increasing incubation time (120 hours), a substantial amount of CH$_3$Hg$^+$ (~43%) was degraded and converted to inorganic Hg (IHg) by *M. trichosporium* OB3b, but not by *M. capsulatus* Bath cell (Fig. 1B). This observation was confirmed by additional detailed time- and concentration-dependent studies of CH$_3$Hg$^+$ degradation by both *M. trichosporium* OB3b (Fig. 2, A and B) and *M. capsulatus* Bath (Fig. 2, C and D). We found no demethylation at all with *M. capsulatus* Bath cultures, regardless of the reaction time (up to 120 hours) and CH$_3$Hg$^+$ concentration (from 5 to 125 nM). However, CH$_3$Hg$^+$ was degraded progressively by *M. trichosporium* OB3b with time and CH$_3$Hg$^+$ concentrations up to 75 nM (Fig. 2, A and B). The pseudo-first-order rate constants at the initial CH$_3$Hg$^+$ concentrations of 5, 25, and 75 nM were 0.017 (±0.001), 0.032 (±0.008), and 0.037 (±0.003) hour$^{-1}$, respectively, and approximately 55, 62, and 73% of the added CH$_3$Hg$^+$ were degraded after 5 days. Again, CH$_3$Hg$^+$ was converted to IHg (Fig. S1A), but no gaseous Hg(0) was observed (fig. S1B). The amount of the cell-associated CH$_3$Hg$^+$, particularly the adsorbed CH$_3$Hg$^+_{\text{ads}}$, decreased with time, whereas the proportion of IHg increased with time. The produced IHg mostly remained inside the cell, with less than 6% of the IHg either left in solution or sorbed on the cell surface because Hg(II) is known to strongly sorb or interact with thiol functional groups of proteins and cellular materials (33). Note that, at the highest added CH$_3$Hg$^+$ concentration (125 nM), the reaction rate decreased to 0.011 (±0.001) hour$^{-1}$ (Fig. 2A), and demethylation was inhibited in the first 8 to 24 hours. However, with a longer incubation time (120 hours), the cells were able to recover and degrade a substantial amount of CH$_3$Hg$^+$ (71%). This initially inhibited CH$_3$Hg$^+$ degradation may be interpreted as a result of potential toxic effects of CH$_3$Hg$^+$ on *M. trichosporium* OB3b, similar to that observed with *Geobacter bemidjiensis* Bem (17).

Because demethylation was observed neither in *M. trichosporium* OB3b spent medium (fig. S1B) nor in *M. capsulatus* Bath cultures (Fig. 2, C and D), the results signify that demethylation was biologically mediated and methanotroph strain specific. However, neither *M. trichosporium* OB3b nor *M. capsulatus* Bath contains a homolog of *M. capsulatus* Bath in 5 mM MOPS buffer. The total added CH$_3$Hg$^+$ concentration (HgT) was 5 nM at **t** = 0, and the cell concentration was 10$^6$ cells ml$^{-1}$. CH$_3$Hg$^+_{\text{ads}}$, soluble CH$_3$Hg$^+$; CH$_3$Hg$^+_{\text{up}}$ cell surface–adsorbed CH$_3$Hg$^+$; CH$_3$Hg$^+_{\text{up}}$, cell uptake of or internalized CH$_3$Hg$^+$. IHg results from degradation of CH$_3$Hg$^+$.

To elucidate this mechanism, we first considered the fact that both *M. trichosporium* OB3b and *M. capsulatus* Bath are sensitive to the availability of copper. That is, the copper-to-biomass ratio is a key factor in regulating the expression of the following: (i) genes encoding for the soluble and particulate methane monooxygenases (MMOs), with soluble MMO only expressed in the absence of copper (25, 34); and (ii) genes encoding for the chalkophore methanobactin with expression greatest in the absence of copper (24, 25). Genes encoding for the chalkophore are found in *M. trichosporium* OB3b, but not in *M. capsulatus* Bath, which contains a different class of chalkophores (35). Furthermore, methanobactin from *M. trichosporium* OB3b has been found to bind Hg(II) and CH$_3$Hg$^+$ (29) and may thus be involved in CH$_3$Hg$^+$ degradation.

We subsequently investigated CH$_3$Hg$^+$ degradation by *M. trichosporium* OB3b in the presence of a known MMO inhibitor, acetylene, but no apparent inhibitory effects were observed (fig. S2). We next considered CH$_3$Hg$^+$ degradation by cells grown either in the absence (0 μM) or in the presence (1 μM) of copper. Although CH$_3$Hg$^+$ degradation was observed under both conditions (Fig. 3 and table S1), greater degradation of CH$_3$Hg$^+$ was evident in the absence than in the presence of copper. We then examined several mutant strains of *M. trichosporium* OB3b defective in methanobactin production (*mbnAN*::Gm$^+$ and Δ*mbnAN*) (25) to determine whether methanobactin is directly involved in CH$_3$Hg$^+$ degradation. We also examined two additional methanotrophs—one (*Methylocystis* strain SB2) makes methanobactin and the other (*Methylocystis parvus* OBBP) does not (36). Results show that *Methylocystis* strain SB2 degraded CH$_3$Hg$^+$, whereas all methanobactin mutants did not regardless of the culture conditions in the presence or absence of Cu$^{2+}$ ions (Fig. 3 and table S1). In addition, no demethylation was observed with *M. parvus* OBBP. These findings strongly suggest that methanobactin plays a critical role in degrading CH$_3$Hg$^+$.

Although methanobactin is clearly needed for CH$_3$Hg$^+$ degradation by *M. trichosporium* OB3b, subsequent studies indicate that it is not sufficient. That is, when CH$_3$Hg$^+$ was incubated with the purified methanobactin, no appreciable CH$_3$Hg$^+$ degradation was observed in the same MOPS buffer used in whole-cell studies (fig. S3). This result suggests that methanobactin likely served as a carrier or as a binding agent for CH$_3$Hg$^+$ in the cell where it is degraded by some as yet unknown mechanisms.
Fig. 2. Time- and concentration-dependent degradation of methylmercury (CH$_3$Hg$^+$) by methanotrophs. (A and B) *M. trichosporium* OB3b at 30°C and (C and D) *M. capsulatus* Bath at 45°C in 5 mM MOPS buffer. The added cell concentration was $10^8$ cells ml$^{-1}$ (washed), and the CH$_3$Hg$^+$ concentration was varied from 0 to 125 nM. Data points at 5 nM CH$_3$Hg$^+$ in (A) represent an average of replicate samples (10 to 15) from five independent batch experiments, and all other data points represent an average of triplicate samples. Error bars represent 1 SD from all replicate samples.

Fig. 3. Methylmercury (CH$_3$Hg$^+$) degradation by different methanotrophs and mutants. Comparisons of the time-dependent degradation of CH$_3$Hg$^+$ by washed cells of *M. trichosporium* OB3b and its mutant strains (mbrA::Gmr and ΔmbrAN), *M. capsulatus* Bath, and *M. parvus* OBBP in 5 mM MOPS buffer. The added cell concentration was $10^8$ cells ml$^{-1}$, and the CH$_3$Hg$^+$ concentration was ~5 nM. Data points represent an average of all replicate samples (3 to 15), and error bars represent 1 SD.
into as yet unknown but potentially widespread biological mechanisms. Hg-resistant bacteria, methanotrophs take up and degrade CH$_3$Hg$^+$ at filtered through 0.2-

addition of methanol (5 mM or higher) completely inhibited CH$_3$Hg$^+$ methanobactin, degraded CH$_3$Hg$^+$, similar to the degradation of methanotrophic-mediated CH$_3$Hg$^+$ degradation was also evident at circumneutral pH, unlike organomercurial lyase that has an optimal pH of ~10 (21). These findings suggest that methanotrophs may play an important role in controlling Hg transformation or net CH$_3$Hg$^+$ production and toxicity in situ, thereby providing new insights into as yet unknown but potentially widespread biological mechanisms of CH$_3$Hg$^+$ uptake and demethylation in the environment.

**MATERIALS AND METHODS**

The methanotrophs _M. trichosporium_ OB3b and _M. capsulatus_ Bath were grown in nitrate minimal salts medium at 30° and 45°C, respectively, either without added copper or with 1 μM copper (as CuCl$_2$) (29, 38). Cells were harvested at the late exponential phase, washed once, and then resuspended in 5 mM MOPS buffer solution at pH 7.3. Methanobactin was isolated from _M. trichosporium_ OB3b, as previously described (39).

Methylmercury (CH$_3$Hg$^+$) sorption, uptake, and demethylation assays were conducted in 4-ml amber glass vials (National Scientific) by mixing washed cells with CH$_3$Hg$^+$ in 5 mM MOPS buffer under ambient conditions. To determine whether MMOs were involved in CH$_3$Hg$^+$ demethylation, we added 100 μl of acetylene to the headspace (through a septum) and allowed it to equilibrate with the cells first for 30 min in one subset of assays because acetylene is a strong and selective inhibitor of MMO activity (25, 40). CH$_3$Hg$^+$ working solution (10 nM) was prepared by diluting 5 μl stock solution (CH$_3$HgOH in 0.5% acetic acid and 0.2% HCl from Brooks Rand Labs) in MOPS. The reaction was initiated by mixing 0.5 ml of CH$_3$Hg$^+$ working solution with 0.5 ml of washed cells to give a final concentration of CH$_3$Hg$^+$ at 5 nM and of cells at 1 × 10$^8$ cells ml$^{-1}$ (17), or otherwise specified. Samples were then placed on a rotary shaker, kept at 30°C for _M. trichosporium_ OB3b and its mutants, and at 45°C for _M. capsulatus_ Bath. Replica sample vials were taken at selected time points and analyzed as follows. For CH$_3$Hg$^+$ sorption (or uptake) analysis, triplicate samples were filtered through 0.2-μm syringe filters (to remove cells) and analyzed for CH$_3$Hg$^+$ uptake (17, 32). The unfiltered samples were used to determine the total Hg and total CH$_3$Hg$^+$ (CH$_3$Hg$^+$$_{total}$) so that the cell-associated or total sorbed Hg can be calculated by their difference. For Hg species distribution analyses, six replicates (in separate vials) were taken, and three of them were filtered as above and analyzed for total soluble Hg (Hg$_{sol}$) and CH$_3$Hg$^+$$_{sol}$ (17, 33). The remaining three samples were used to determine cellular uptake of CH$_3$Hg$^+$ (CH$_3$Hg$^+$$_{up}$) and cell-surface-adsorbed CH$_3$Hg$^+$ (CH$_3$Hg$^+$$_{ads}$). This was accomplished by adding 2,3-dimercapto-1-propanesulfonic acid (DMPS), a Hg-chelating agent, at 150 μM to wash off the sorbed CH$_3$Hg$^+$$_{sol}$ at each time point and then analyzing CH$_3$Hg$^+$$_{sol}$ in filtered samples (17, 33), so that CH$_3$Hg$^+$$_{up}$ can be calculated by subtracting CH$_3$Hg$^+$$_{sol}$ and CH$_3$Hg$^+$$_{sol}$ from CH$_3$Hg$^+$$_{total}$. The inorganic Hg species, resulting from degradation of CH$_3$Hg$^+$, were analyzed in the same manner, in which the adsorbed Hg$_{sol}$ and cellular uptake of Hg$_{up}$ were determined following DMPS washing, and soluble Hg (Hg$_{sol}$) was calculated by subtracting CH$_3$Hg$^+$$_{sol}$ and Hg$_{sol}$ (17, 33). Selected samples (before filtration) were determined for purgeable elemental Hg(0), but none was detected. Additional experiments were performed with cell spent medium and MOPS buffer as controls. Demethylation experiments were repeated at least once to ensure data quality, and error bars in all figures represent 1 SD of all replicate samples. Demethylation rate constants (kdemeth) were calculated on the basis of the pseudo-first-order rate law: [d(CH$_3$Hg$^+$)/dt]$_{demeth}$ = -kdemeth[CH$_3$Hg$^+$], where k$_{demeth}$ was determined by the slope of the linear regression between natural logarithm of the CH$_3$Hg$^+$ concentration and time (12, 41).

A modified EPA Method 1630 was used for CH$_3$Hg$^+$ analysis, in which isotope dilution with enriched CH$_3$Hg$^+$$_{200}$ was used as an internal standard, and an inductively coupled plasma mass spectrometer (ELAN DRC-e, PerkinElmer Inc.) was used to separate the various Hg isotopes to determine CH$_3$Hg$^+$ concentrations (17, 32, 33). The recovery of spiked CH$_3$Hg$^+$ standards was 100 ± 10%, and the detection limit was about 3 × 10$^{-5}$ nM CH$_3$Hg$^+$. Gaseous Hg(0) was directly determined by inserting needles through the septa of the 4-ml glass vials and then purging with ultrapure N$_2$ for 2 min into a gaseous Hg(0) analyzer (Lumex 915+, Ohio Lumex). Total Hg and Hg$_{sol}$ were analyzed via SnCl$_2$ reduction and detection by the Lumex analyzer after samples were oxidized in BrCl (5%, v/v) overnight at 4°C (11, 12, 42). The detection limit was ~2.5 × 10$^{-4}$ nM.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/5/e1700041/DC1
table S1. Methylmercury degradation by washed cells of methanotrophs, including _M. trichosporium_ OB3b and its two methanobactin (mb) defective mutants (mmbA::Gmr and ∆mmbAN). _Methylcystis_ strain SB2, _M. capsulatus_ Bath, and _M. parvus_ OB3B in 5 mM MOPS buffer at pH 7.3. Methylmercury (CH$_3$Hg$^+$) and inorganic mercury (Hg) species distribution during CH$_3$Hg$^+$ degradation assays with _M. trichosporium_ OB3b.

tab. S2. Effects of acetylene addition (as an inhibitor of MMOs) on methylmercury (CH$_3$Hg$^+$) degradation by washed cells of _M. trichosporium_ OB3b (10$^8$ cells ml$^{-1}$) in 5 mM MOPS buffer at 30°C. Reactions between methylmercury (CH$_3$Hg$^+$), 5 mM and purified methanobactin (1 μM) from _M. trichosporium_ OB3b in 5 mM MOPS buffer (pH 7.3) at 30°C.

**REFERENCES AND NOTES**


Acknowledgments: We thank X. Yin for Hg and MeHg analyses, Y. Liu and Z. Yang for technical assistance and discussion, and A. Johs for critical review of the manuscript. Funding: This research was sponsored by the U.S. Department of Energy (DOE) Office of Science, Office of Biological and Environmental Research, as part of the Mercury Science Focus Area at the Oak Ridge National Laboratory, which is managed by UT-Battelle, LLC under contract no. DE-AC05-00OR22725 with DOE and grant no. DE-SC0006630 to J.D.S. and A.A.D. Author contributions: X.L., B.G., J.D.S., and A.A.D. designed the research. X.L., W.G., I.Z., and M.F.U.H. performed the experiments and analyses. B.G., J.D.S., and A.A.D. wrote the paper. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 4 January 2017
Accepted 30 March 2017
Published 31 May 2017
10.1126/science.a1700041

Methylmercury uptake and degradation by methanotrophs
Xia Lu, Wenyu Gu, Linduo Zhao, Muhammad Farhan Ul Haque, Alan A. DiSpirito, Jeremy D. Semrau and Baohua Gu

Sci Adv 3 (5), e1700041.
DOI: 10.1126/sciadv.1700041